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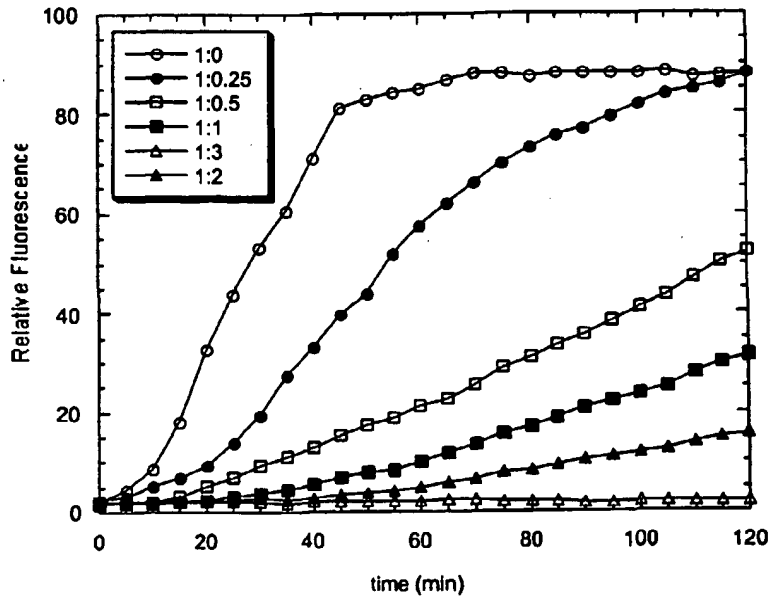
(54) Title: USE OF MATRIX METALLOPROTEINASE PEPTIDE SUBSTRATE TO LOWER THE RATE OF EXTRACELLULAR MATRIX TURNOVER



(57) Abstract: The present invention relates to novel peptides and methods for enhancing wound healing, especially chronic wounds. The peptides of the present invention act as substrates for proteinases found in wounds, such as matrix metalloproteinases (MMPs) and human neutrophil elastase. Tailoring of the peptide sequences proveds control of the healing process. The invention also relates to methods of treating wounds and inhibiting degradation of collagen, elastase, stromelysin, and other proteins dound in wounds.

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USE OF MATRIX METALLOPROTEINASE PEPTIDE SUBSTRATES TO LOWER THE RATE OF EXTRACELLULAR MATRIX TURNOVER

FIELD OF THE INVENTION

The present invention relates to compositions and methods for enhancing wound healing, especially chronic wounds (e.g., diabetic wounds, pressure sores). More specifically, the invention relates to improved wound healing through regulation of matrix metalloproteinase activity.

BACKGROUND OF THE INVENTION

In normal tissues, cellular connective tissue synthesis is offset by extracellular matrix degradation, the two opposing effects existing in dynamic equilibrium. Degradation of the matrix is brought about by the action of matrix metalloproteinases (MMPs) released from resident connective tissue cells and invading inflammatory cells. Normally, these catabolic enzymes are tightly regulated at the level of their synthesis and secretion and also at the level of their extracellular activity. Extracellular control occurs primarily by regulation with specific regulatory proteins, such as TIMPs (tissue inhibitors of metalloproteinases), which form complexes with MMPs. These complexes prevent MMP action. Cellular level control of MMP activity occurs primarily by regulating MMP gene expression and by down

regulating the expression of the membrane bound MMPs (MT-MMP) that activate the excreted proenzyme form of the MMP.

MMPs are a family of natural metalloenzymes capable of degrading extracellualr matrix (ECM) macromolecules. There are currently approximately 23 accepted members of the MMP enzyme family, including membrane-bound forms. Members of this family that have been isolated and characterized include interstitial fibroblast collagenase, stromelysin, and type IV collagenase. Other potential members include a poorly characterized 94,000 dalton gelatinase and several low molecular weight gelatinases and telopeptidases. Structurally, MMPs contain a catalytic zinc(II) site at the active site of the protein. A bound catalytic zinc is required for hydrolytic activity.

TIMPs are glycoproteins that specifically regulate interstitial collagenase on a 1:1 stoichiometric basis. That is, TIMPs form very specific regulatory complexes with MMPs, only regulating a specific subset of the MMPs. No naturally occurring TIMP molecule singly regulates all types of MMPs.

In chronic wounds, the ratio of MMPs to TIMPs is high, such that most of the MMPs are unregulated. This unregulated MMP activity results in the accelerated, uncontrolled breakdown of the ECM, leading to destruction of the newly formedwound bed. Additionally, the concomitant elevation of proteinase levels, cause hydrolyzation of TIMP molecules, further increasing the MMP to TIMP ratio.

Many individuals suffer from chronic wounds. Open cutaneous wounds represent one major category of such wounds and include burn wounds, neuropathic ulcers, pressure sores, venous stasis ulcers, and diabetic ulcers. Worldwide, eight million people have chronic leg ulcers and seven million people have pressure sores (Clinica 559, 14-17, 1993). In the U.S. alone, the prevalence of skin ulcers is 4.5 million, including two million pressure sore patients, 900,000 venous ulcer patients and 1.6 million diabetic ulcer patients (Med Pro Month, June 1992, 91-94). The cost involved in treating these wounds is staggering and, at an average of $3,000 per patient, reaches over $13 billion per year for the U.S. alone.

Burn wounds have a reported incidence of 7.8 million cases per year worldwide, 0.8 million of which need hospitalization (Clinica 559). In the U.S., there are 2.5 million burn patients per year, 100,000 of which need hospitalization and 20,000 of which have burns involving more than 20% of the total body surface area (MedPro Month, June 1992).

Many other problems also result from the uncontrolled breakdown of connective tissues by MMPs. These problems include, for example, rheumatoid arthritis; osteoarthritis; osteopenias, such as osteoporosis, periodontitis, gingivitis, corneal epidermal, and gastric ulceration; tumour metastasis, invasion, and growth; neuroinflammatory disorders, including those involving myelin degradation, for example,

multiple sclerosis; and angiogenesis dependent diseases, which include angiofibromas, hemangioma, solid tumors, blood-borne tumors, leukemia, metastasis, telangiectasia, psoriasis, scleroderma, pyogenic granuloma, myocardial angiogenesis, plaque neovascularization, coronary collaterals, ischemic limb angiogenesis, corneal diseases, rubeosis, neovascular glaucoma, diabetic retinopathy, retrolental fibroplasia, arthritis, diabetic neovascularization, macular degeneration, wound healing, peptic ulcer, fractures, keloids, vasculogenesis, hematopoiesis, ovulation, menstruation, and placentation.

Given the large number of diseases associated with MMP activity, there is a need to control MMP activity. Several approaches have been suggested to accomplish such regulation. One approach has focused on the catalytic role of zinc in MMPs, designing zinc-chelating regulators. Potent regulators have been generated by introducing zinc chelating groups, such as peptide hydroxamates and thiol-containing peptides, into substrates. Peptide hydroxamates and TIMPs have been successfully used in animal models to treat cancer and inflammation. While these hydroxamates are potent at regulators of MMPs by binding to zinc, they are toxic to humans because they bind to all zinc-containing enzymes. Because many biochemical reactions occurring in the body require zinc, use of the hydroxamates detrimentally effects these other functions and can result in death.

Other known zinc-chelating MMP regulators are peptide derivatives based on naturally occurring amino acids and are analogues of the cleavage site in the collagen molecule (Odake *et al.* (1994) Biophys. Res. Comm. 199, 1442-46). Some MMP regulators are less peptidic in structure and may more properly be viewed as pseudopeptides or peptide mimetics. Such compounds usually have a functional group capable of binding to the zinc (II) bound in the MMP. Known compounds include those in which the zinc binding group is a hydroxamic acid, carboxylic acid, sulphydryl, or oxygenated phosphorus (for example, phosphinic acid and phosphonamidate, including aminophosphonic acid) groups.

Other approaches include small molecule regulation (Levy *et al.* (1998) J. Med. Chem. 41, 199-223; Wojtowicz-Praga *et al.* (1997) Invest. New Drugs 15, 61-75; Duivenvoorden, *et al.* (1997) Invasion and Metas. 17, 312-22) and regulation via anti-MMP antibodies (Su *et al.* (1995) Hybridoma. 14, 383-90).

More specifically, an elastase inhibitor is disclosed in U.S. Patent No. 5,734,014 to Ishima *et al.* Elastase secreted by neutrophils causes tissue damage, and in this process, creates an active abundance of oxygen. Elafin isolated from psoriatics has elastase inhibiting activity. However, this naturally occurring elafin is unstable to oxidation. *Ishima* developed elafin derivatives that are stable to oxidation so that elastase regulation can be more efficient. The oxidation-stable derivative is

created by partly modifying the amino acid sequence of natural elafin. The modification can be created by either chemical synthesis or site-directed mutagenesis.

U.S. Patent No. 5,464,822 to Christophers *et al.* discloses a polypeptide that possesses inhibitory activity against human leukocyte elastase. The polypeptides possess inhibitory activity that is specific for serine proteases. For example, they possess inhibitory activity against proteases, such as human leukocyte elastase and porcine pancreatic elastase, but do not possess any significant inhibitory activity against trypsin. These polypeptides can be prepared by genetic engineering or obtained from psoriatic scales of human skin.

U.S. Patent No. 5,698,671 to Stetler-Stevenson *et al.* discloses a protein defined by the presence of specific cysteine-containing amino acid sequences, isolated from the conditioned media of cultured human tumor cells, that binds with high affinity to MMPs and analogs thereof. The particular inhibitor is made by preparing peptides and proteins having a cysteine residue at the same interval as that of the various tissue inhibitors of metalloproteinase (TIMPs). The peptides must have at least two appropriately spaced cysteines to ensure inhibitory activity by virtue of a disulfide bridge formation. In addition, the invention discloses a method for purifying natural MMP inhibitors by MMP affinity chromatography.

Despite these varied approaches, the current art does not selectively regulate MMP activity. Traditionally, high affinity regulators have been utilized, resulting in complete MMP inhibition. However, shutting off all MMP activity is actually deleterious to the healing process, as some MMP activity is required for tissue remodelling. For example, potent inhibition aimed at binding the zinc (II) site is toxic to humans because it shuts off bind to all zinc-containing enzymes. It is therefore necessary to have regulation be selective.

Thus, there is a need in the art for improved regulation of MMPs to promote healing of chronic and acute wounds.

There is also a need in the art for an inhibitor having relatively good affinity, which is selective.

Furthermore, there is a need in the art for MMP inhibitors that are not toxic to the individual to whom they are adminisered.

SUMMARY OF THE INVENTION

The present invention comprises substrate peptides that enhance the healing of wounds, especially chronic wounds. These peptides interact with the active sites on proteinases that degrade the proteins present in the wound site. The proteinases are responsible for the reorganization of the ECM that is necessary for wound healing and include matrix metalloproteinases and human neutrophil elastase. The

substrate peptides of the invention compete with the natural proteins for proteinase binding.

The present invention also comprises compositions containing the substrate peptides and uses of the peptides and compositions for treating chronic wounds. These peptides provide a reversible method for regulating proteinase activity and improving wound healing. The amount of peptide administered and the particular design of the peptide used can provide different degrees of regulation of ECM degradation and reorganization.

Further, the present invention comprises methods for developing synthetic substrate peptides having amino acid sequences that bind to the active sites of the proteinases present in wounds. The peptides can be designed to include sequences that bind to only one of the proteinases or that bind to multiple proteinases. In addition, peptides can be designed that inhibit some proteinases, allowing other proteinases to remain active. In this way the degree and specificity of regulation of ECM degradation can be controlled.

Thus, it is an object of the invention to provide substrate peptides that include amino acid sequences that bind to the active site of one or more proteinases, such as MMPs and hNEs.

It is another object of the present invention to provide substrate peptides that hydrolyze proteinases that regulate wound healing and/or ECM degradation and reorganization.

It is also an object of the present invention to provide substrate peptides having SEQ ID Nos. 1-20.

It is another object of the present invention to provide a method for regulating the degradation and reorganization of the ECM.

It is a further object of the invention to provide a method for inhibiting the degradation of collagen.

It is an object of the present invention to provide a method for inhibiting the degradation of elastin, fibrin, and other proteinaceous materials degraded by elastase.

It is also an object of the present invention to provide a method for inhibiting degradation of proteinaceous substances degraded by stromelysin.

It is another object of the present invention to provide a method of treating wounds that does not introduce potentially toxic organic molecules into the wound site.

It is a further object of the invention to provide peptides that do not adversely affect patient physiology outside the wound bed.

It is an object of the present invention to provide a method for regulating the activity of proteinases.

It is another object of the present invention to provide a method for selectively regulating the activity of one or more specific MMPs.

It is a further object of the present invention to provide compositions that comprise one or more of the substrate peptides of the invention.

It is an object of the present invention to provide wound dressings that comprise the substrate peptides and allow for extended release of the peptides from the dressing.

It is another object of the invention to provide compositions containing the substrate peptides in the form of lotions, ointments, creams, gels, sprays, foams, solutions, emulsions, and the like.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates a time course of collagen degradation in the presence of varying amounts of the substrate peptide having SEQ ID No. 1. Fluoresceinated collagen (substrate) was simultaneously mixed with MMP-9 and the peptide at the noted molar stoichiometries. The assay measured the release of fluorescence containing collagen fragments (excitation wavelength 490 nm, emission wavelength 520 nm)

as a function of time. The legend denotes the enzyme to peptide molar stoichiometry.

Figure 2 depicts fluoresceinated collagen mixed with MMP-9 (2.5 μg) at time zero. Emission intensity at 520 nm was continuously measured (excitation wavelength 480 nm). At 2200 seconds, the peptide havinf SEQ ID No. 1 was added to the reaction (at a MMP-9: peptide ratio of 1:3). No correction for dilution had been performed. The assay showed the reduced rate of collagen hydrolysis (2200-4600 seconds) during which the peptide was preferentially degraded, followed by the return of pre-addition collagen hydrolysis kinetics.

Figure 3 represents a time course of collagen degradation in the presence of varying amounts of the dual substrate peptide having SEQ ID No. 2. Fluoresceinated collagen (substrate) was simultaneously mixed with human neutrophil elastase and the peptide at the noted molar stoichiometries (denoted as enzyme to peptide on the graph). The assay measured the release of fluorescence containing collagen fragments (excitation wavelength 490 nm, emission wavelength 520 nm) as a function of time.

Figure 4 illustrates a time course of collagen degradation in the presence of varying amounts of the dual substrate peptide having SEQ ID No. 2. Fluoresceinated collagen (substrate) was simultaneously mixed with MMP-9 and the peptide at the noted molar stoichiometries (denoted as enzyme to peptide on the graph). The assay measured the

release of fluorescence containing collagen fragments (excitation wavelength 490 nm, emission wavelength 520 nm) as a function of time.

Figure 5 shows a fluoresceinated collagen mixed with MMP-9 (1.0 μM) and human neutrophil elastase (1.0 μM) at time zero. Emission intensity at 520 nm was continuously measured (excitation wavelength 480 nm). At 500 seconds (first arrow), the peptide: Pro-Leu-Gly-Leu-Ala-Ala-Pro-Gly-Val-Tyr was added to the reaction (at a total enzyme to peptide ratio of 1:5). No correction for dilution had been performed. The assay showed the reduced rate of collagen hydrolysis (1,000-10,000 seconds) during which time the peptide was preferentially degraded. At 3500 seconds and again at 7000 seconds, additional enzyme (0.5 μM of each was added).

Figure 6 depicts compound viability assays. The graph plots the percent viability of the peptides utilized in this study relative to a PBS control. Error bars are +/-SD. Samples (left to right) are as follows: PBS positive control, 1%Triton X-100 negative control, PepSub-1, PepSub-2, PepSub-3.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention comprises substrate peptides that enhance the healing of wounds, especially chronic wounds. These peptides interact with the active sites on proteinases that degrade the proteins present in the wound site. The proteinases are responsible for

the reorganization of the ECM that is necessary for wound healing and include matrix metalloproteinases and human neutrophil elastase. The substrate peptides of the invention compete with the natural proteins for proteinase binding.

Matrix metalloproteinases are enzymes that degrade the proteins found in the ECM. They include, but are not limited to, collagenases, elastases, stromelysins, and gelatinases. In chronic wounds and other disease states these MMPs and other proteinases are present in excess. This excess of MMPs inhibits healing of the wound through increased the breakdown of the ECM.

In one aspect, the present invention comprises substrate peptides that compete with the natural proteins, such as collagen, to reduce the number of free proteinases, such as MMPs and hNEs, available to interact with the natural proteins in the ECM. The substrate peptides of the present invention are tailored to provide improved affinity over the natural proteins. Thus, the proteinases in the wound exudate preferentially bind to the substrate peptides of the invention over the natural proteins present in the wound site. The MMPs. and other proteinases are then hydrolyzed.

The present invention comprises the design of specific substrate peptides that can be tailored to provide varying specificity, affinity, and hydrolyzation rates for individual proteinases. Substrate peptides as used herein refers to any peptide containing a linear

combination of amino acids that can be cleaved by any proteinase found in chronic wounds.

In one embodiment, the substrate peptide can be designed so that it binds to the active site of only one MMP. Alternatively, the substrate protein can be designed so that it contains amino acids that bind to the active sites of multiple proteinases. In this manner, the level of some MMPs can be lowered, while the levels of other MMPs are left unaffected. For example, the substrate peptide can bind only MMP9 leaving the other proteinases in the wound unaffected and available for interaction with the natural peptides. Alternatively, it is possible to simultaneously divert MMPs 3, 8, and 9, while leaving MMP1 free to interact with the natural peptides. Since MMPs interact with different substrate proteins and provide different functions in wound healing, this approach provides substrate proteins that can inhibit detrimental proteinases while enhancing levels of those that aid in the healing process.

In another embodiment, the present invention comprises the design of substrate peptides that lower the levels of different proteinases at differing levels. For example, a particular substrate peptide of the invention can lower MMP9 three fold, MMP8 five fold, and MMP3 two fold. The ability to provide this degree of specificity is a significant improvement over conventional methods of wound treatment.

Any substrate peptide that can interact with one or more of the proteinases present in chronic wounds to inhibit its ability to bind the natural peptides present in the wound can be used in the present invention. Effective sequence variations are almost unlimited. Preferred subtrate peptides include, but are not limited to, those having SEQ ID Nos. 1-20 (Table 1 below). As noted in the Table, some of these substrate peptides interact with only one type of protein, e.g. SEQ ID No. 3, while others interact with multiple proteins, e.g. SEQ ID No. 1. In addition to the peptides listed below, peptides containing linear combinations of these peptides are useful in the present invention for binding to different proteinases or different binding sites on a single proteinase. The term linear combination means that the entire sequence, e.g. SEQ ID No.3, is attached to the end of another entire sequence, e.g. SEQ ID No. 9. Further, active fragments of the peptides can be used in the present invention. All that is required is that the active fragment bind a proteinase present in the wound site such that it cannot interact with the natural peptides present in the wound.

Table 1. Substrate Peptides

Collagenases	
SEQ ID No. 3	Pro-Leu-Gly-Leu-Leu-Gly
SEQ ID No. 4	Pro-Leu-Gly-Pro-Arg
SEQ ID No. 5	Pro-Gln-Gly-Ile-Ala-Gly-Gln-Arg
SEQ ID No. 6	Pro-Leu-Gly-Cys-His-Ala-Arg
SEQ ID No. 7	Pro-Leu-Gly-Leu-Trp-Ala-Arg
SEQ ID No. 8	Pro-Leu-Gly-Leu-Tyr-Ala-Arg
Elastases	

SEQ ID No. 9	Ala-Ala-Ala
SEQ ID No. 10	Ala-Ala-Pro-Ala
SEQ ID No. 11	Ala-Ala-Pro-Val
SEQ ID No. 12	Ala-Pro-Ala
SEQ ID No. 13	Ala-Ala-Pro-Leu
SEQ ID No. 14	Ala-Ala-Pro-Phe
SEQ ID No. 15	Ala-Tyr-Leu-Val
Stromelysins	
SEQ ID No. 16	Pro-Leu-Gly-Leu-Trp-Ala-Arg
SEQ ID No. 17	Arg-Pro-Lys-Pro-Tyr-Ala-Val-Trp-Met-Lys
SEQ ID No. 18	Pro-Leu-Gly-Leu-Ala-Ala-Arg
Mixed substrate peptides	
SEQ ID No. 19	Pro-Leu-Gly-Met-Trp-Ser-Arg
SEQ ID No. 20	Arg-Pro-Lys-Pro-Val-Glu-Val-Trp-Arg
SEQ ID No. 1	Arg-Pro-Lys-Pro-Tyr-Ala-Val-Trp-Met-Lys
SEQ ID No. 2	Pro-Leu-Gly-Leu-Ala-Ala-Pro-Gly-Val-Tyr

In another embodiment, the substrate peptides of the present invention can be modified. For example, the substrate peptides of the invention can be modified by cyclization, N-terminal acetylation, C-terminal carboxylation, and other such modifications.

In another aspect, the present invention comprises compositions containing these substrate peptides and uses of the peptides and compositions for treating chronic wounds. The compositions comprise one or more of the substrate peptides and a pharmaceutically acceptable carrier. The compositions can be in the form of a lotion, cream, ointment, gel, foam, spray, paste, granules, powder, solution, dispersion, emulsion, or the like. The compositions can also contain

other ingredients, such as excipients, emollients, time release agents, and other active ingredients.

In one embodiment, one or more of the substrate peptides of the invention can be incorporated into a time release composition to provide for the extended release of the peptides into the wound bed. In another embodiment, the peptides or compositions can be incorporated into wipes, bandages, or wound dressings. For example, the peptides or compositions can be incorporated into wound dressings, such as hydrogels, so that they are released over time into the wound exudate. In yet another embodiment, the peptides can be incorporated into a spray or foam that can be sprayed directly onto the wound, or can be applied to bandages prior to placement over the wound.

In another aspect, the present invention comprises methods for treating chronic wounds, and for the treatment of other diseases or conditions for which MMP activity is associated. These methods comprise administering to the wound or other affected area one or more of the substrate peptides of the invention. The preferred dose range for administration is between about 10 μg and 1 mg, more preferably 100 μg to 0.5 mg. The peptides can also be administered in the compositions of the invention. In another embodiment, the peptides can be applied *in vitro* for the regulation of MMPs in synthetic models to study wound healing.

Particular embodiments of the invention will be now be discussed in more detail. These embodiments are simply examples of the invention and are in no way limiting as to the scope of the invention. In one such embodiment of the present invention, the decapeptide having SEQ ID No. 1 is introduced into the wound bed. The peptide is preferably introduced in molar excess, yet it is effective at sub stoichiometric amounts. Furthermore, the overall activity of MMP-9 against a collagen substrate can be titrated effectively simply by varying the concentration of the peptide. With this type of substrate competition strategy, a fine degree of control over proteinase level is possible. As shown in Figure 1, this inhibits the MMP-9 catalyzed hydrolysis of fluoresceinated collagen.

As shown in Figure 2, the substrate peptide of the present invention can prevent collagen destruction for a significant period of time even in the presence of both MMP-9 and human neutrophil elastase. A typical MMP-9 reaction begins at time zero and proceeds until 2200 seconds, at which time a three fold molar excess of the peptide having SEQ ID No. 1 is introduced into the assay cuvette. During the time period to 4500 seconds, the peptide is preferentially hydrolyzed. Once the peptide pool is completely hydrolyzed, collagen destruction resumes. This "protection period" can be manipulated by changing the peptide concentration or the number and type of hydrolysis recognition sites in the peptide. Figure 2 also demonstrates that in this protection window, there is still a slight amount of proteinase catalyzed

hydrolysis of collagen. This is important for the ECM reorganization that is needed in a pro-healing process.

In another embodiment, proteinase activity if non-MMP enzymes that are found in the wound bed can be diverted away from collagen. Desirably, the peptide having SEQ ID No. 2 is added to the wound bed. The rate of human neutrophil elastase catalyzed collagen destruction can be greatly reduced by the addition of this peptide in super stoichiometric amounts, however, it is still effective in molar excess. As is shown in Figure 3, even substoichiometric amounts of the competitive substrate significantly reduce collagen hydrolysis.

In another embodiment of this invention, a single peptide that contains a hydrolysis site for each proteinase in the chronic wound environment can be added to the wound bed. Further, the number of such individual sites to the relative concentration of that proteinase in wound exudates can be tailored. This approach optimizes nearest neighbor sequences and avoids the need to introduce multiple peptides into the wound. One such multi-site peptide, SEQ ID No. 2, contains hydrolysis sites for both MMP-9 and human neutrophil elastase (hNE). This peptide can reduce the amount of collagen degradation in the simultaneous presence of MMP-9 and hNE. As illustrated in Figure 4, such multi-substrate peptides are just as effective in diverting proteinase activity from a collagen substrate in mixed enzyme assays, as are single site peptides in single enzyme systems.

SEQ ID No. 19 is also capable of continued collagen protection when challenged with the addition of new proteinase. Figure 5 illustrates that the protection window can be maintained over a period of three hours, even when fresh MMP-9 and hNE are added to the assay. Upon the addition of fresh enzyme (at 3500 and 7000 seconds) there is a slight increase in the rate of collagen destruction, followed by a resumption of collagen protection. Since the biosynthesis of proteinases is continuous in a chronic wound, that is new proteinase is constantly introduced into the wound environment, this experiment is meant to simulate this feature of wound dynamics. SEQ ID No. 19 at suitable concentrations is able to divert newly introduced enzyme from collagen hydrolysis. K_{mapp} values, apparent K_m values were calculated from the fluorescence data are shown in Table 2.

Table 2.

Peptide	Sequence	$K_{mapp}(\mu M)$
PepSub-1	SEQ ID No. 1	3.2
PepSub-2	SEQ ID No. 2	0.6
PepSub-3	SEQ ID No. 19	5.7

A feature of many proteinase inhibitors is their relative toxicity. It is preferred that a skin equivalent toxicity model (Epiderm) is employed to measure the overall cellular viability in the presence of peptide constructs. Specifically, a single dose of 10 mM peptide (in PBS) is applied to the Epiderm samples for a period of 12 hours. As per

the present invention, the resulting viability is plotted in Figure 6. A PBS control is set to a value of 100 percent viability. The surfactant Triton X-100 served as a negative control, that is the application of a 1% triton solution should result in over 90% cell death. As can be seen in Figure 6, the peptides of the invention have an overall higher viability of 99.7 +/- 2.8 percent.

The present invention is further illustrated and supported by the following examples. However, these examples should in no way be considered to further limit the scope of the invention. To the contrary, one having ordinary skill in the art would readily understand that there are other embodiments, modifications, and equivalents of the present invention without departing from the spirit of the present invention and/or the scope of the appended claims

EXAMPLES

BASIC PROCEDURES

Peptide Synthesis

All peptides were synthesized by Sigma-Genosys, Inc. The released peptides were purified to >95% homogeneity via RP-HPLC by the company. The pooled eluted peak material was desalted and lyophilized. Mass Spec analysis confirmed the peptide molecular weight and purity. Unless otherwise noted, all chemicals were purchased from

Sigma Chemical Corp. or from Fluka Chemical Co. Active MMP-9 enzyme was purchased from Calbiochem.

Molecular Modeling

Molecular modeling utilized two visualization programs, Swiss PDB Viewer (Guex and Peitsch, 1997) and Rasmol (Sayle and Milner-White, 1995). Model work was performed on a Compaq PC running Windows 95, as well as a Silicon Graphics, Inc. Octane UNIX workstation. Additionally, the Cerius2 molecular package from Molecular Simulations, Inc. was utilized on the Octane. Three dimensional structure files were downloaded from the Protein Databank as follows (filename, reference): MMP-1 (1FBL, Li *et al.*, 1995), MMP-2 (1GEN, Libson *et al.*, 1995), MMP-8 (1JAO, 1JAN, Grams, *et al.*, 1995; Reinemer *et al.*, 1994), MMP-9 (1MMQ, Browner *et al.*, 1995), TIMP-2/MT-1 MMP complex (1BUV, Fernandez-Catalan *et al.*, 1998), TIMP-2 (1BR9, Tuuttila *et al.*, 1998), and TIMP-1/MMP complex (1UEA, Gomis-Ruth *et al.*, 1997; Huang *et al.*, 1996; Becker *et al.*, 1995). These files were used to analyze the three-dimensional structure of the proteins, and the chemical nature and identification of conserved and variant amino acids in the MMP-TIMP contact interface, as well as to inspect the amino acid composition and nature of the various MMP active sites.

Assay Procedure

The assay measured the enzymatic hydrolysis of fluoresceinated collagen by MMP9 as a function of time. Fluoresceinated collagen (Molecular Probes, Inc.) at a concentration of 5 mM was added to one of the following reaction buffers: 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 5 mM $CaCl_2$, and 0.1 mM NaN_3. This solution was placed into a Spectrosil quartz fluorometer cuvette. MMP at a concentration of 0.1 mM was mixed with varying amounts of substrate peptide having SEQ ID No. 1-3 and incubated at 25°C for 10 minutes to effect binding. The protein mixture was added to the collagen substrate and mixed quickly. Fluorescence emission intensity at 520 nm was measured as a function of time (excitation wavelength 495 nm) in a Shimadzu RF5301 fluorometer. The fluorescein release assay was used to determine the apparent K_m (K_{mapp}) of the substrate peptide [ps] according to Segel (1993) via the use of 1/v vs 1/[S] plots, such that the replotted slopes from each the reciprocal plot lines (in a summation slope vs. [ps] plot gives:

$$\text{slope} = K_{mapp}/V_{max} \quad (1)$$

where K_m is the Michaelis constant, V_{max} is the reaction maximum velocity, v is the instantaneous velocity, and [S] and [ps] are the substrate and substrate peptide concentrations, respectively.

Example 1

The decapeptide having SEQ ID No. 1 (PepSub-1) in varying amounts was simultaneously mixed with fluoresceinated collagen (substrate) and MMP-9 at molar stoichiometries of 1:0, 1:0.25, 1:0.5, 1:1, 1:2, and 1:3. The assay measured the release of fluorescence containing collagen fragments (excitation wavelength 490 nm, emission wavelength 520 nm) as a function of time. The legend denotes the enzyme to peptide molar stoichiometry. The results of this assay are shown in Figure 1.

Example 2

Fluoresceinated collagen was mixed with MMP-9 (2.5 μg) at time zero. Emission intensity at 520 nm was continuously measured (excitation wavelength 480 nm). At 2200 seconds, the peptide having SEQ ID No. 1 was added to the reaction (at a MMP-9:peptide ratio of 1:3). No correction for dilution was been performed. The assay showed the reduced rate of collagen hydrolysis (2200-4600 seconds), during which the peptide was preferentially degraded, followed by the return of pre-addition collagen hydrolysis kinetics. The results of this assay are shown in Figure 2.

Example 3

A time course of collagen degradation was performed. Human neutrophil elastase and fluoresceinated collagen (substrate) were simultaneously mixed with the dual substrate peptide having SEQ ID

No. 2 in varying amounts. The molar stoichiometries of enzyme:peptide were 1:0, 1:0.5, 1:1, and 1:2. The assay measured the release of fluorescence containing collagen fragments (excitation wavelength 490 nm, emission wavelength 520 nm) as a function of time. The results of this assay are shown in Figure 3.

Example 4

A time course of collagen degradation was performed. Fluoresceinated collagen (substrate) was simultaneously mixed with MMP-9 and the dual substrate peptide having SEQ ID No. 2. The stoichiometric amounts of MMP:peptide are 1:1, 1:2, and 1:3. The assay measured the release of fluorescence containing collagen fragments (excitation wavelength 490 nm, emission wavelength 520 nm) as a function of time. The results are shown in Figure 4.

Example 5

Fluoresceinated collagen is mixed with MMP-9 (1.0 μM) and human neutrophil elastase (1.0 μM) at time zero. Emission intensity at 520 nm is continuously measured (excitation wavelength 480 nm). At 500 seconds (first arrow), the peptide having SEQ ID No. 2 was added to the reaction (at a total enzyme to peptide ratio of 1:5). No correction for dilution had been performed. The assay showed the reduced rate of collagen hydrolysis (1,000-10,000 seconds) during which time the

peptide is preferentially degraded. At 3500 seconds and again at 7000 seconds, additional enzyme (0.5 μM of each) was added. The results are shown in Figure 5.

Example 6

A skin equivalent toxicity model (Epiderm) was employed to measure the overall cellular viability in the presence of the substrate peptides having SEQ ID Nos. 1-3. A single dose of 10 mM peptide (in PBS) was applied to the Epiderm samples for a period of 12 hours. The resulting viability is plotted in Figure 6. A PBS control was set to a value of 100 percent viability. The surfactant Triton X-100 served as a negative control. As can be seen in Figure 6, all three peptides exhibit an overall viability of 99.7 +/- 2.8 percent. .

CLAIMS

WE CLAIM:

1. A method of inhibiting ECM degradation comprising administering to the ECM one or more substrate peptides, wherein the substrate peptide competes with the ECM for proteinaceous activity.

2. A method of inhibiting ECM degradation comprising administering to the ECM one or more substrate peptides comprising one or more of SEQ ID Nos. 1-20.

3. The method of Claim 2 wherein the substrate peptide comprises one or more of SEQ ID Nos. 1, 2, 19, or 20.

4. A method of inhibiting the degradation of collagen comprising administering to a collagen substrate one or more substrate peptides, wherein the substrate peptide competes with collagenase activity.

5. A method of inhibiting the degradation of collagen comprising administering to a collagen substrate one or more substrate peptides comprising one or more of SEQ ID Nos. 3-8.

6. A method of inhibiting the degradation of elastin, fibrin, or other proteinaceous substances degraded by elastase comprising administering to the substance one or more substrate peptides, wherein the substrate peptide competes with the substance for elastase activity.

7. A method of inhibiting the degradation of elastin, fibrin, or other proteinaceous substances that are degraded by elastase comprising administering to the substance one or more substrate peptides comprising one or more of SEQ ID Nos. 9-15.

8. A method of inhibiting the degradation of proteinaceous materials that are degraded by stromelysin comprising administering to the material one or more substrate peptides, wherein the substrate peptide competes with the substrate for stromelysin activity.

9. A method of inhibiting the degradation of proteinaceous materials that are degraded by stromelysin comprising administering to a stromelysin substrate one or more substrate peptides comprising one or more of SEQ ID Nos. 16-18.

10. A method of treating wounds comprising administering to an individual having a wound an effective amount of one or more substrate peptides, wherein the peptide comprises one or more of SEQ ID Nos. 1-20.

11. The method of Claim 10 wherein the substrate peptide comprises one or more of SEQ ID Nos. 1, 2, 19, or 20.

12. The method of Claim 10 wherein the substrate peptide comprises one or more of SEQ ID Nos. 3-8.

13. The method of Claim 10 wherein the substrate peptide comprises one or more of SEQ ID Nos. 9-15.

14. The method of Claim 10 wherein the substrate peptide comprises one or more of SEQ ID Nos. 16-18.

15. The method of Claim 10 wherein the wound is a chronic wound.

16. The method of Claim 10 wherein the substrate peptide is administered in an amount between 10 μg and 1 mg.

17. The method of Claim 10 wherein the substrate peptide is administered in a wound dressing.

18. The method of Claim 10 wherein the substrate peptide is administered in a composition.

19. The method of Claim 18 wherein the composition provides for release of the peptide over time.

20. A method of treating wounds comprising administering to an individual having a wound an effective amount of a substrate peptide that competes with natural proteins in the wound for proteinaceous activity.

21. The method of Claim 20 wherein the natural proteins are selected from the group consisting of collagen, elastin, fibrin, other ECM proteins.

22. The method of Claim 20, wherein the proteinases are selected from MMPs.

23. The method of Claim 20 wherein the proteinase is human neutrophil elastase.

24. The method of Claim 20 wherein the wound is a chronic wound.

25. The method of Claim 20 wherein the substrate peptide is administered in an amount between 10 μg and 1 mg.

26. The method of Claim 20 wherein the substrate peptide is administered in a wound dressing.

27. The method of Claim 20 wherein the substrate peptide is administered in a composition.

28. The method of Claim 27 wherein the composition provides for release of the peptide over time.

29. A substrate peptide comprising one or more of SEQ ID Nos. 1-20.

30. The substrate peptide of Claim 20 comprising SEQ ID No. 1.

31. The substrate peptide of Claim 20 comprising SEQ ID No. 2.

32. The substrate peptide of Claim 20 comprising SEQ ID No. 3.

33. The substrate peptide of Claim 20 comprising SEQ ID No. 4.

34. The substrate peptide of Claim 20 comprising SEQ ID No. 5.

35. The substrate peptide of Claim 20 comprising SEQ ID No. 6.

36. The substrate peptide of Claim 20 comprising SEQ ID No. 7.

37. The substrate peptide of Claim 20 comprising SEQ ID No. 8.

38. The substrate peptide of Claim 20 comprising SEQ ID No. 9.

39. The substrate peptide of Claim 20 comprising SEQ ID No. 10.

40. The substrate peptide of Claim 20 comprising SEQ ID No. 11.

41. The substrate peptide of Claim 20 comprising SEQ ID No. 12.

42. The substrate peptide of Claim 20 comprising SEQ ID No. 13.

43. The substrate peptide of Claim 20 comprising SEQ ID No. 14.

44. The substrate peptide of Claim 20 comprising SEQ ID No. 15.

45. The substrate peptide of Claim 20 comprising SEQ ID No. 16.

46. The substrate peptide of Claim 20 comprising SEQ ID No. 17.

47. The substrate peptide of Claim 20 comprising SEQ ID No. 18.

48. The substrate peptide of Claim 20 comprising SEQ ID No. 19.

49. The substrate peptide of Claim 20 comprising SEQ ID No. 20.

50. A composition comprising one or more substrate peptides, wherein the substrate peptide comprises SEQ ID Nos. 1-20.

51. The composition of Claim 50 wherein the composition is in the form of a lotion, cream, ointment, gel, spray, foam, paste, granules, powder, solution, dispersion, or emulsion.

52. A composition comprising one or more substrate peptides, wherein the substrate peptide competes with ECM proteins for proteinaceous activity.

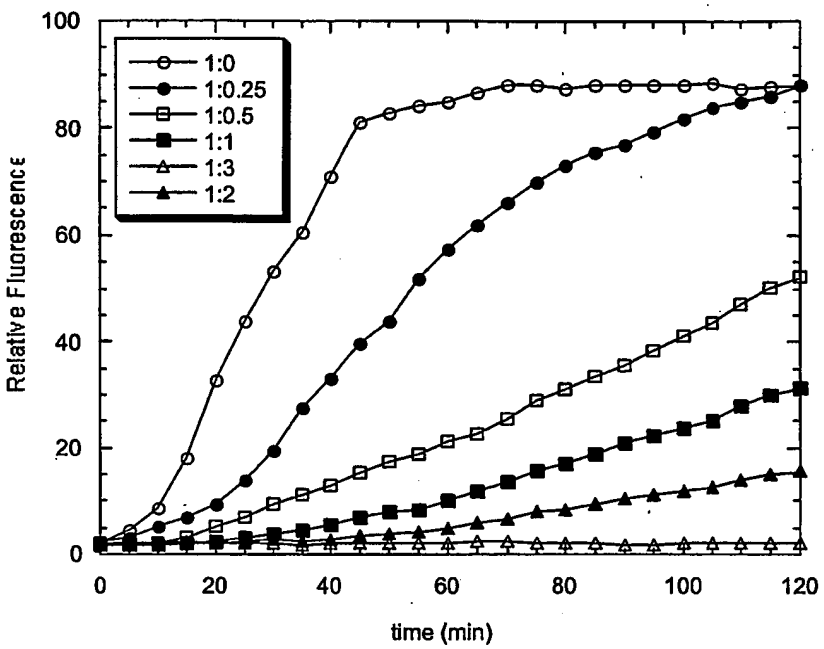
53. The composition of Claim 52 wherein the composition is in the form of a lotion, cream, ointment, gel, spray, foam, paste, granules, powder, solution, dispersion, or emulsion.

54. The composition of Claim 52 wherein the ECM protein is collagen.

55. A wound dressing comprising one or more substrate peptides or a composition thereof.

56. The wound dressing of Claim 55 wherein the substrate peptide is a peptide that competes with ECM proteins for proteinaceous activity or a composition thereof.

57. The wound dressing of Claim 55 wherein the substrate peptide is a peptide comprising one or more of SEQ ID Nos. 1-20.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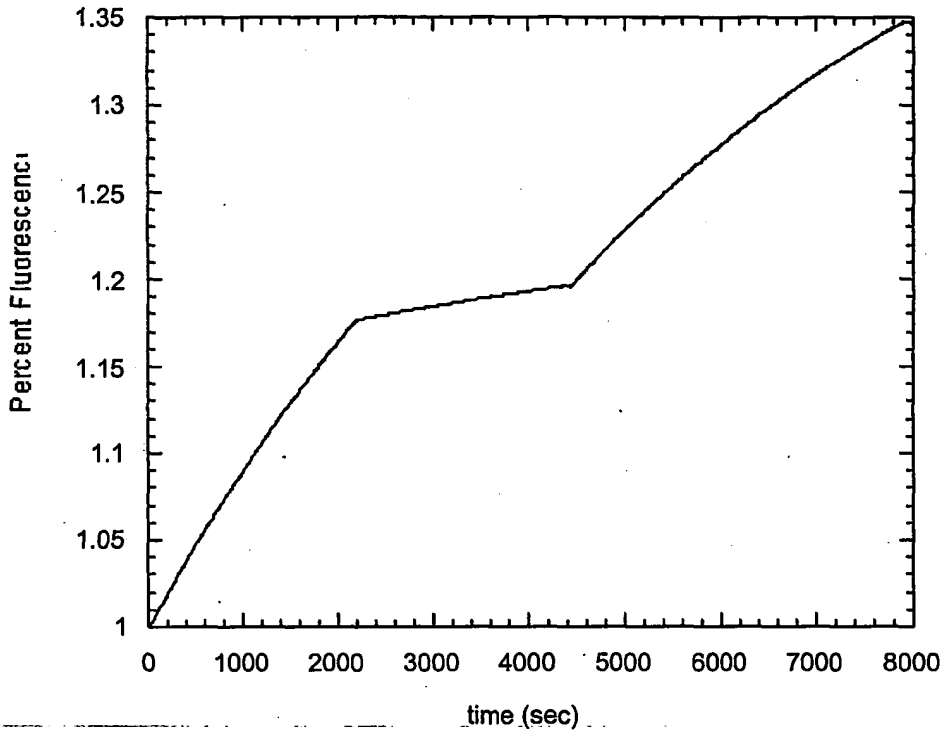
1:0.5
1:1
1:3
1:2

Figure 1


1.35
1.3
1.25
1.2
1.15
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Percent Fluorescenc
0
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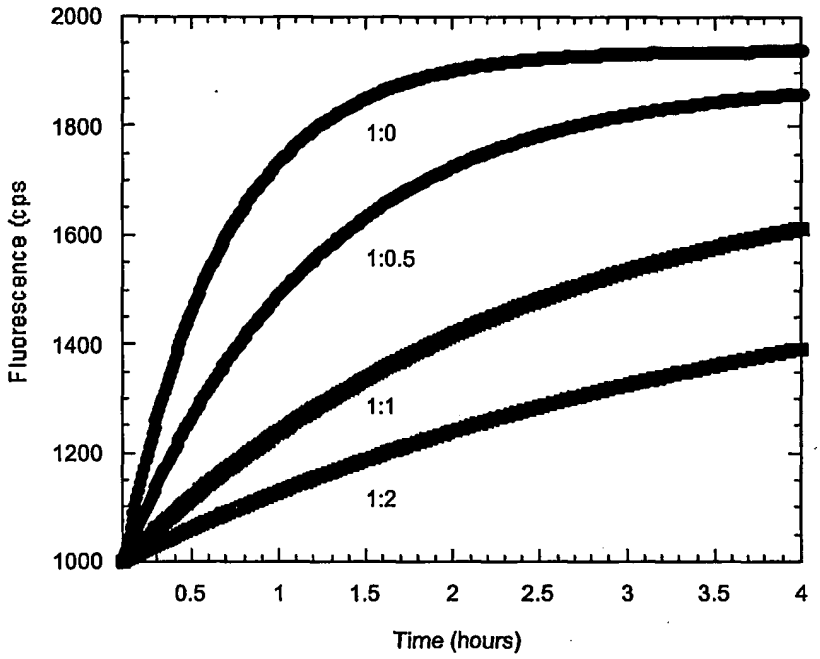
3000
4000
5000
6000
7000
8000
time (sec)

Figure 2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1400
1200
1000
Fluorescence (cps
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1:2
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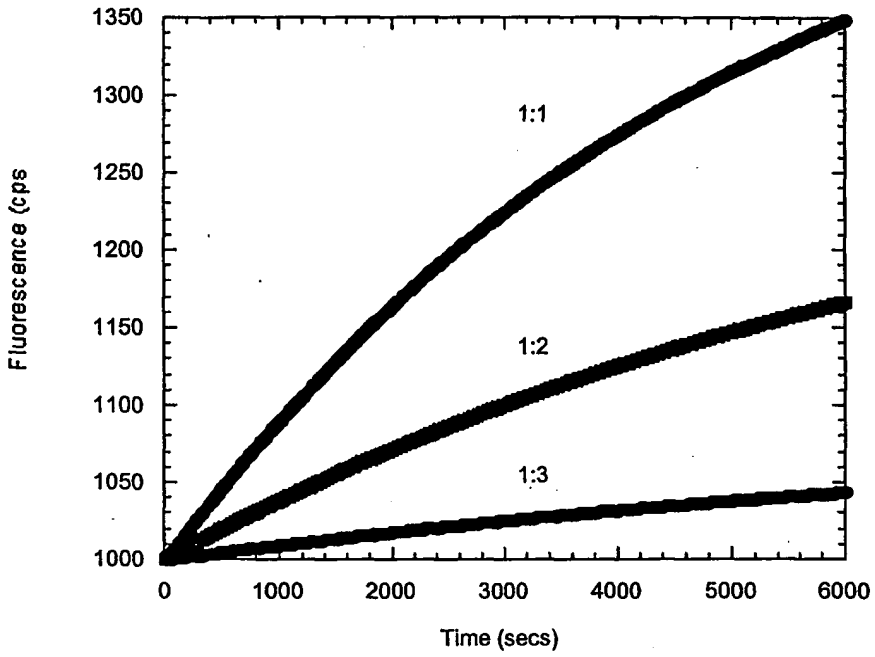
1.5
2
2.5
3
3.5
4
Time (hours)

Figure 3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1050
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1:2
1:3
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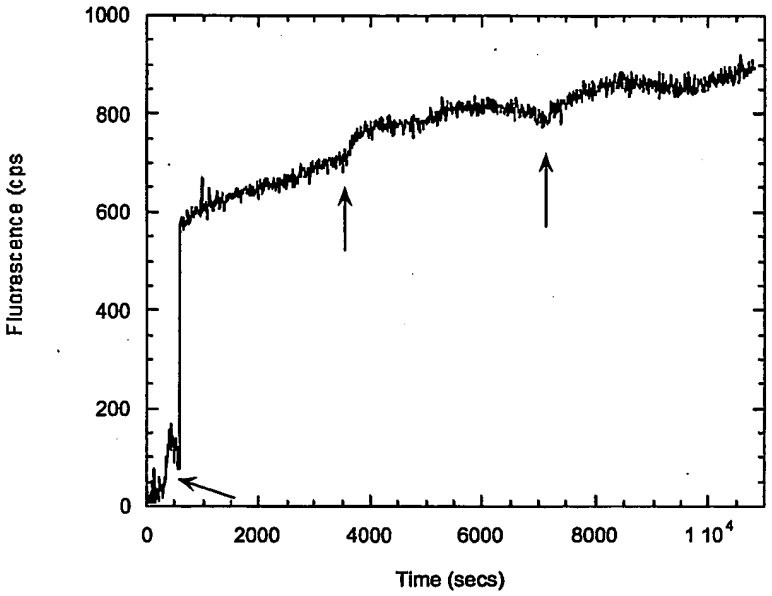
3000
4000
5000
6000
Time (secs)

Figure 4: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6000
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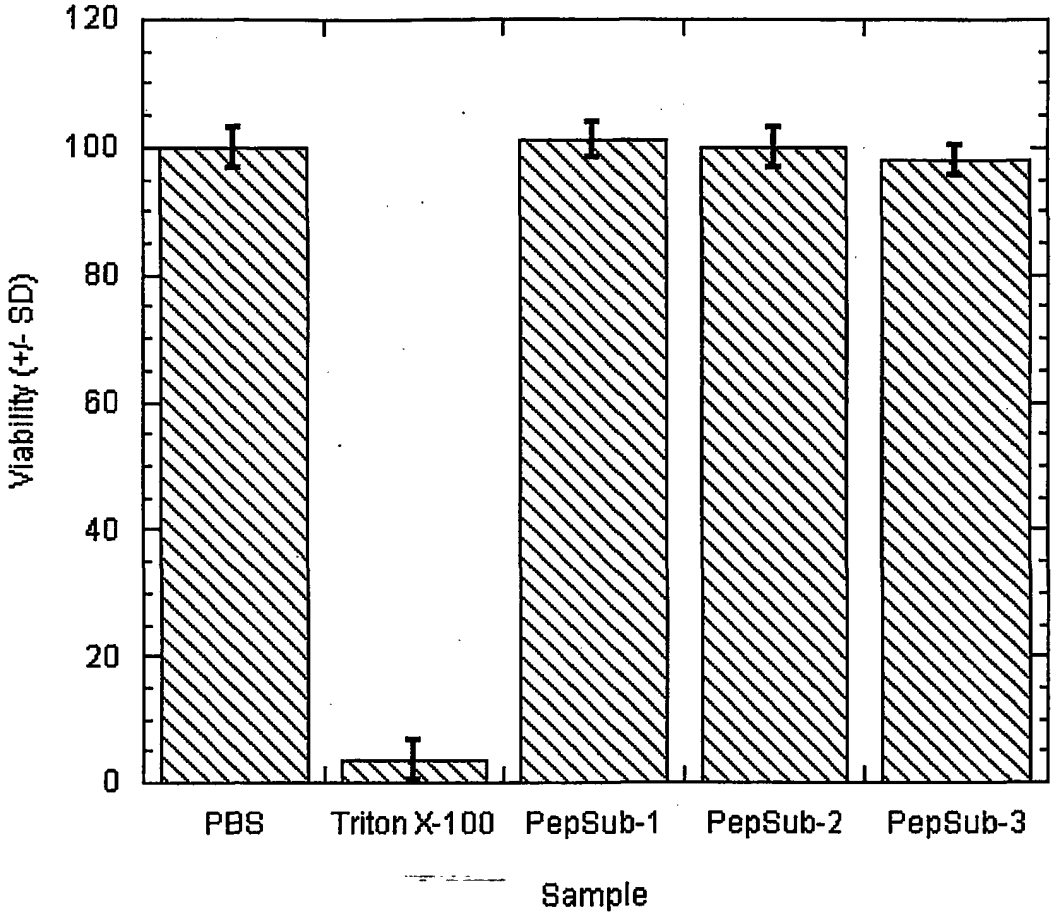
1 10^4
Time (secs)

Figure 5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100
80
60
40
20
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Viability (+/- SD)
PBS
Triton X-100
PepSub-1
PepSub-2
PepSub-3
Sample

Figure 6

SEQUENCE LISTING

<110> Quirk, Stephen

McGrath, Kevin

<120> Use of Matrix Metalloproteinase Peptide Substrates to Lower the Rate of Extracellular Matrix Turnover

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<160> 20

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1 5

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(71) Applicant: KIMBERLY-CLARK WORLDWIDE, INC. [US/US]; 401 North Lake Street, Neenah, WI 54956 (US).

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(74) Agents: PRATT, John, S. et al.; Kilpatrick Stockton LLP, Suite 2800, 1100 Peachtree Street, Atlanta, GA 30309 (US).

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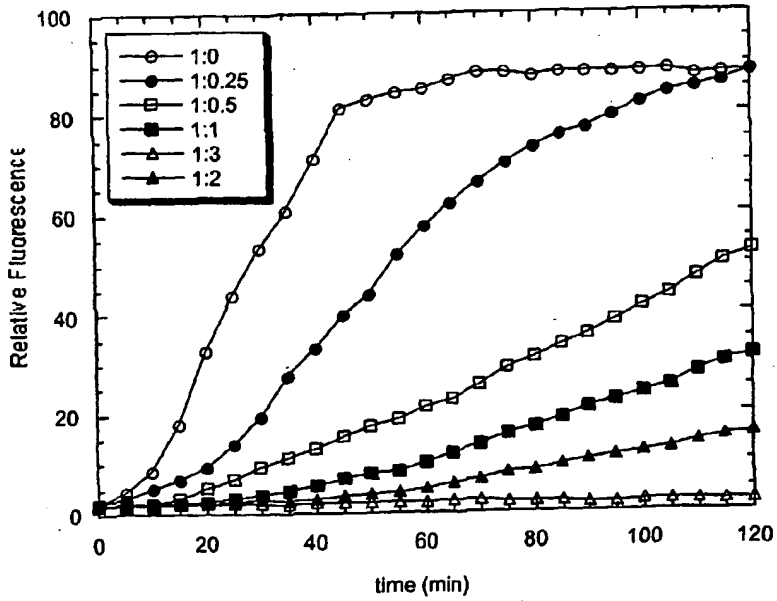
Published:
— *with international search report*

[Continued on next page]

(54) Title: USE OF MATRIX METALLOPROTEINASE PEPTIDE SUBSTRATE TO LOWER THE RATE OF EXTRACELLULAR MATRIX TURNOVER



(57) Abstract: The present invention relates to novel peptides and methods for enhancing wound healing, especially chronic wounds. The peptides of the present invention act as substrates for proteinases found in wounds, such as matrix metalloproteinases (MMPs) and human neutrophil elastase. Tailoring of the peptide sequences proveds control of the healing process. The invention also relates to methods of treating wounds and inhibiting degradation of collagen, elastase, stromelysin, and other proteins dound in wounds.

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(88) Date of publication of the international search report:
26 February 2004

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 01/49272

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K5/08 C07K5/10 C07K7/06 A61K38/06 A61K38/07
A61K38/08 A61P17/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, CHEM ABS Data, EMBASE, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
E	WO 02 38108 A (THE J. DAVID GLADSTONE INSTITUTES) 16 May 2002 (2002-05-16) claims 1-31 ---	42,43, 50-54
X,P	WO 01 46220 A (POLYMUN SCIENTIFIC IMMUNBIOLOGISCHE FORSCHUNG GMBH) 28 June 2001 (2001-06-28) page 4, line 32 claims 1-17 ---	38,50-54
X	WO 00 63233 A (UNIVERSITY OF FLORIDA RESEARCH FOUNDATION INC. ET AL) 26 October 2000 (2000-10-26) claims 1-3,13-15 --- -/--	41,50-54

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11 March 2003	18/03/2003

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Siatou, E

Form PCT/ISA/210 (second sheet) (July 1992)

International Application No
PCT/US 01/49272

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 618 790 A (JAMES C. KENNEDY ET AL) 8 April 1997 (1997-04-08) column 5, line 64 - line 67 column 6, line 15 - line 17 column 6, line 44 - line 48	33,34, 50-54
X	US 5 612 194 A (H. RUBIN ET AL) 18 March 1997 (1997-03-18) column 13 -column 14	40,43
X	US 5 534 496 A (V. H. LEE ET AL) 9 July 1996 (1996-07-09) claims 1-8	33,50-54
X	GB 2 131 813 A (MONSANTO COMPANY) 27 June 1984 (1984-06-27) claims 1-8	32,50-54
X	EP 0 126 009 A (CENTRE NATIONALE DE LA RECHERCHE SCIENTIFIQUE (CNRS)) 21 November 1984 (1984-11-21) claims 1-13	6,52,53

INTERNATIONAL SEARCH REPORT

national application No.
PCT/US 01/49272

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 1-9, insofar as in vivo applications are concerned, as well as claims 10-28 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [X] Claims Nos.: 1, 4, 6, 8, 20-28, 52-56
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

see FURTHER INFORMATION sheet PCT/ISA/210

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 1, 4, 6, 8, 20-28, 52-56

Present claims 1, 4, 6, 8, 20-28, 52-56 relate to an extremely large number of possible compounds and methods. Support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT is to be found, however, for only a very small proportion of the compounds and methods claimed. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Consequently, the search has been carried out for those parts of the claims which appear to be supported and disclosed, namely those parts relating to the compounds and methods using the substrate peptides with SEQ. ID Nos 1-20.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

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